

1 **Identification of natural mutations responsible for altered infection phenotypes of**
2 ***Salmonella enterica* clinical isolates using cell line infection screens**

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26 Abstract

27 The initial steps of *Salmonella* pathogenesis involve adhesion and invasion of host epithelial
28 cells. While well-studied for *S. Typhimurium*, the factors contributing to this process in other,
29 host-adapted serovars remains unexplored. Here, we screened clinical isolates of serovars
30 Gallinarum, Dublin, Choleraesuis, Typhimurium and Enteritidis for adhesion and invasion of
31 intestinal epithelial cell lines of human, porcine, and chicken origins. 30 isolates with altered
32 infectivity were used for genomic analyses and 14 genes and novel mutations associated with
33 high or low infectivity were identified. The functions of candidate genes included virulence
34 gene expression regulation, cell wall or membrane synthesis and components. The role of
35 several of these genes in *Salmonella* adhesion and invasion to cells has not previously been
36 investigated. The genes *dksA* (stringent response regulator) and *sanA* (vancomycin high-
37 temperature exclusion protein) were selected for further analyses, and we confirmed their
38 roles in host cell adhesion and invasion. Furthermore, transcriptomic analyses were performed
39 for *S. Enteritidis* and *S. Typhimurium*, with two highly infective and two marginally infective
40 isolates for each serovar. Expression profiles for the isolates with altered infection phenotypes
41 revealed the importance of T3SS expression levels in the determination of isolate's infection
42 phenotype. Taken together, these data indicate a new role in cell host infection for genes or
43 gene variants previously not associated with adhesion and invasion to the epithelial cells.

44

45 Importance

46 *Salmonella* is a foodborne pathogen affecting over 200 million people and resulting in over
47 200,000 fatal cases per year. Adhesion to and invasion of *Salmonella* into intestinal epithelial
48 cells is one of the first and key steps in the pathogenesis of salmonellosis. Still, around 35-40%
49 of bacterial genes have no experimentally validated function and their contribution to the
50 bacterial virulence, including adhesion and invasion, remains largely unknown. Therefore the

51 significance of this study is in the identification of new genes or gene allelic variants
52 previously not associated with adhesion and invasion. It is well established that blocking
53 adhesion and/or invasion would stop or hamper bacterial infection, therefore the new findings
54 from this study could be used in future developments of anti-*Salmonella* therapy targeting
55 genes involved in these key processes. Such treatment could be a valuable alternative as the
56 numbers of antibiotic-resistant bacteria is growing very rapidly.

57 **Keywords:** *Salmonella*, adhesion, invasion, infection, clinical isolates, epithelial cells,
58 *sanA*, *dksA*, genomics, transcriptomics

60 1. Introduction

61
62 Typhoidal and non-typhoidal salmonellosis is one of the major causes of foodborne
63 diseases globally, affecting over 200 million people and resulting in over 200,000 fatal cases
64 per year (1, 2). Animal salmonellosis is of considerable importance not only because of
65 animal infection, but also as a reservoir for human infections (3). The causative agent in both
66 humans and animals is *Salmonella enterica* subsp. *enterica*, which is comprised of more than
67 1500 serovars, of which only a few are of clinical significance (4). Based on clinical
68 presentation and host-range, *Salmonella* serovars can be classified as either host-restricted,
69 host-adapted and host-unrestricted (5). Most serovars from clinical samples are host-
70 unrestricted and are able to infect multiple hosts, usually resulting in gastroenteritis (6). Host-
71 adapted ‘specialists’ like *S. Dublin* and *S. Choleraesuis* mainly cause systemic disease in
72 cattles and pigs, respectively, but can sporadically cause systemic infections in other hosts
73 (e.g. human) (7, 8). Host-restricted serovars are limited to a single host where they cause
74 systemic infections, for example *S. Gallinarum* (poultry) or *S. Typhi* (human and primates) (1,
75 9).

76 As a facultative intracellular, gastrointestinal pathogen, the initial steps of *Salmonella*
77 pathogenesis involve adhesion to and invasion of host intestinal epithelial cells (10).
78 *Salmonella* has evolved various strategies for adhesion and invasion, and different serovars
79 have developed specific combinations of various adhesins or variants of the same adhesin for
80 host cell attachment (11, 12). Adhesive structures of *Salmonella enterica* can be divided into
81 three groups: fimbrial adhesins, non-fimbrial adhesins, and atypical adhesive structures (13).
82 The role of the majority of these in the pathogenesis of various *Salmonella* serovars remains
83 unknown. *Salmonella* invasion of host cells is mediated by the type three secretion system
84 (T3SS) encoded on the *Salmonella* pathogenicity island 1 (SPI-1) (14), although additional
85 factors such as the proteins Rck and PagN have also been found to play a role (15, 16).
86 Despite an overwhelming amount of research and a seemingly clear picture of the adhesion
87 and invasion process, largely based on work with the serovar *S. Typhimurium*, our
88 understanding of these phenomena is still not complete. For example, it is now evident that
89 adhesion and invasion can be affected by expression of different adhesin variants, mutations
90 in genes encoding T3SS effectors, environmental factors, stress, metabolism, and various
91 regulatory proteins (12, 17). Additionally, around 35-40% of bacterial genes have no
92 experimentally validated function and their contribution to the bacterial virulence, including
93 adhesion and invasion, remains unknown (18). Therefore the search for new genes involved in
94 these processes is highly desirable.

95 To find new virulence factors contributing to adhesion or invasion, in this study we
96 investigated the adhesion and invasion of various *Salmonella* serovars against model cell lines
97 derived from three host species: human Caco-2, porcine IPEC-J2 and chicken CHIC-8E11.
98 The study included host-restricted *S. Gallinarum* (from chicken), host-adapted *S. Dublin*
99 (from cattle) and *S. Choleraesuis* (from various animals, largely swine) and host-unrestricted
100 *S. Typhimurium* (from humans and pigs) and *S. Enteritidis* (from humans and chicken). For

101 quantification of adherent and invasive *Salmonella in vitro*, we used a previously established
102 VideoScan technology (19, 20) with a new VideoScan module specifically developed for
103 high-throughput quantification of *Salmonella* infected cells. Moreover, using whole genome
104 sequencing, we identified mutations affecting adhesion and invasion in novel genes, for which
105 contributions to adhesion and invasion were further validated. Additionally, we used RNAseq
106 to identify the differentially expressed genes affecting adhesion and invasion. This study
107 improves the understanding of mechanisms underlying adhesion and invasion of host
108 epithelial cells during *Salmonella* infection and shows contribution of small genome
109 alterations to the bacterial virulence.

110

111 2. Materials & methods

112 2.1. Bacterial isolates and plasmids

113 All bacterial isolates used in this study (Table 1) were tested for ampicillin (Amp) and
114 kanamycin (Kan) resistance with agar disc diffusion tests. For ampicillin-sensitive isolates,
115 plasmid pFPV25.1, containing an Amp resistance gene and the GFPmut3 fluorescent protein
116 expressed from the constitutive *rpsM* promoter, was used (21) . To exchange the Amp
117 resistance gene to a Kan resistance gene for those isolates that were ampicillin-resistant but
118 kanamycin-sensitive, Gibson assemble reaction was performed (NEB). The primers that were
119 used for the amplification of pFPV25.1 plasmid and Kan resistance cassette are listed in Table
120 2. PCR was performed with the use of Phusion High-Fidelity DNA Polymerase (Thermo
121 Scientific), according to the manufacturer's protocol. Gibson assemble was performed
122 according to the manufacturer's protocol. The new plasmid was named pFPV25.1Kan.

123 For adhesion and invasion screening, all 127 *Salmonella* isolates were GFP-labelled by
124 transformation with pFPV25.1 or pFPV25.1Kan. Electrocompetent bacteria were prepared as
125 described previously by (Sambrook and Russell, 2006) with some modifications. After

126 transformation with plasmid, single colonies were re-streaked on fresh Luria-Bertani (LB)
127 agar plates (with appropriate antibiotics). Cultures were prepared in 1 ml of LB (with
128 appropriate antibiotics) in 2-ml Eppendorf tubes and grown for 16 h, and glycerol-based
129 cryostocks were prepared from these cultures.

130 2.2. Cell culture

131 Three intestinal epithelial cell lines were used in this study: porcine IPEC-J2 (obtained from
132 Peter Schierack, (22)), human Caco-2 (DMSZ, Germany) and chicken CHIC-8E11
133 (MicroMol GmbH, obtained from Karsten Tedin, FU Berlin). Cells were grown in D-
134 MEM/Ham's F-12 (Millipore) supplemented with 1 mM L-glutamine and
135 penicillin/streptomycin (Millipore) and either 5% (IPEC-J2 and CHIC-8E11) or 10% (Caco-
136 2) bovine serum (Millipore), and were incubated at 37°C with 5% CO₂ and passaged using
137 standard protocols. For infection assays IPEC-J2, Caco-2 and CHIC-8E11 were seeded in
138 Nunclon Delta 96-well plates (Nunc) at a density of 0.7x10⁴, 1x10⁴ and 2x10⁴, respectively
139 and used after 5 days. For gentamycin protection assay, Caco-2 cells were seeded in 24-well
140 plates (Nest) at a density 5.8x10⁴ and used after 5 days.

141 2.3. VideoScan module for cell line infection assays

142 For automated bacterial quantification, we further developed our VideoScan technology (23)
143 by creating a new module. Briefly, the VideoScan hardware consists of an inverse
144 epifluorescence Olympus microscope with multiple fluorescence channels, a digital camera
145 and a motorized scanning stage. The VideoScan module works with a 20x magnification
146 objective and first focuses on DAPI-stained cell nuclei in a well, which are then marked as
147 position 0 nm. Afterwards the software takes a Z-stack of images (for IPEC-J2, CHIC-8E11, 6
148 images; Caco-2, 7 images) of bacteria (GFP stained) with a starting position of -2000 nm (for
149 IPEC-J2, CHIC-8E11) or 0 nm (for Caco-2). Next, one composite image is made from the Z-

150 stack images taken and bacteria on the image are detected and counted. Twenty (20)
151 composite images per well were analysed during read-out.

152

153 **2.4. Determination of the linear range of the VideoScan assay**

154 To test the reliability of our automated bacteria quantification assay, *S. Typhimurium* SL1344,
155 transformed with a pFPV25.1Kan plasmid, was grown to late exponential phase ($OD_{600}=0.95$ -
156 1.0 , 2.85×10^8 of bacteria/ml) at 37°C and 180 rpm and washed with Phosphate-Buffered
157 Saline (PBS). After this, 50 μl of bacteria were applied in a dilution series (2×10^4 - 3×10^7 /well;
158 final concentration of bacteria- 4×10^5 - 6×10^8 /ml) on plates with a monolayer of IPEC-J2,
159 Caco-2 and CHIC-8E11 cells. Bacteria were incubated for 1 h and the plates were washed and
160 the underlying cell line monolayer with bound bacteria was fixed with 4% paraformaldehyde
161 (PFA, Roth). Cell nuclei were stained with DAPI. Next, adhered bacteria in the plates were
162 counted using the VideoScan module. Three independent experiments with three repetitions
163 for each dilution were prepared and measured.

164

165 **2.5. Cell line infection assays**

166 To screen the 127 *Salmonella* isolates for infectivity, cell lines were seeded in 96-well
167 plates and assayed on day 5, 6 and 7 after seeding. 127 *Salmonella* isolates with GFP
168 expression were used for infection assays. *S. Typhimurium* SL1344 was used as a standard
169 strain in all infection assays. Bacteria were grown in 1 ml of LB (with 50 $\mu\text{g/ml}$ of Kan or 100
170 $\mu\text{g/ml}$ of Amp) in 2-ml Eppendorf tubes at 37°C and 180 rpm. Before an assay, bacteria were
171 diluted to a concentration of 4×10^7 bacteria/ml and 50 μl of bacteria were applied per well to a
172 monolayer of IPEC-J2, Caco-2 and CHIC-8E11 cells. Bacteria were incubated for 1 h and 4 h
173 in a cell culture incubator and the plates were washed three times with PBS. Cell lines with
174 bound bacteria were fixed with 4% PFA in PBS, and the plates were washed with PBS and

175 cell line nuclei were stained with DAPI. Afterwards, adhered bacteria were counted with the
176 VideoScan. Three independent experiments with three repetitions for each *Salmonella* isolate
177 was performed.

178

179 **2.6. Next-generation genome sequencing and comparative genome analysis**

180 Genomic DNA was isolated with the Wizard® Genomic DNA Purification Kit (Promega)
181 according to the manufacturer's instruction. DNA was sequenced with the use of HiSeq X
182 platform at Sanger Institute Sequencing Facility. Sequencing quality was assessed on the basis
183 of average base quality, GC content and adapter contamination (24). Genomes were
184 assembled using the Shovill pipeline and assembly_improvement pipeline (25). Genome
185 assemblies were annotated with Prokka (26). Pangenomes were determined by using Roary
186 (27). Core genome phylogeny was performed with FastTree 2.1 software using generalized
187 time-reversible model of nucleotide evolution. The FastTree 2.1 produces results similar to
188 Maximum Likelihood analysis and the accuracy and reproducibility of this method has been
189 estimated (28). Phylogenetic trees were annotated with iTOL software (29). Comparison of
190 gene presence between isolates with different infection phenotypes was performed with
191 Scoary (30). Multilocus sequence types were determined with the MLST 2.1 online tool and
192 the presence of pSV plasmid was checked with the PlasmidFinder (31, 32).

193 For single nucleotide polymorphism search, the reads obtained for each isolate were
194 mapped against a reference genome using bwa (33) (Supplementary Table 1). Mapped reads
195 were individually analysed with Artemis (34). All isolates belonging to the same serovar were
196 compared with a reference genome from the same serovar. The mutation found in each
197 genome was considered as potentially associated with altered infection phenotypes if it was
198 deletion or single nucleotide substitution which resulted in premature stop codons and/or was
199 placed in the gene with confirmed contribution to adhesion or invasion.

200

201 **2.7. Bacterial mutant construction**

202 For *S. Enteritidis* clinical isolates no. 5706 and 6203, scarless and markerless negative
203 selection-based system was utilized to cure the identified mutations of interest as in the
204 publication of Khetrpal et al. (2015) (35). Primers used for mutant generation are listed in
205 Table 2. To determine whether the isolates showed altered morphologies in comparison to the
206 parental isolates, cultures (grown for 16 h) were stained with acridine orange and investigated
207 with the use of a fluorescence microscope.

208 **2.8. Growth curves**

209 Overnight (O/N) cultures (grown for 16 h) or exponential growth cultures were diluted to
210 5×10^6 bacteria/ml in LB, antibiotic-free cell culture medium or M9 minimal medium with 0.2
211 % glucose (for strain 5706) and 200 μ l of bacterial suspension was pipetted to each well of
212 96-well polystyrene plate. Bacterial growth was measured with a Tecan plate reader for 16 h
213 with 30 seconds of vigorous shaking and OD₆₀₀ measurement every 15 minutes. Three
214 technical and biological repetitions were performed for each strain. Negative controls were
215 included for each medium used in each assay. To assess differences in growth of 5706 isogenic
216 strains, the package Growthcurver was used and growth rate (r), doubling time (t_{gen}) and
217 empirical area under curve (AUC) were used for comparisons between strains (36).

218 Growth with vancomycin for strain 6203 was performed with the use of O/N cultures
219 (grown for 16 h). Bacteria were diluted in Müller-Hinton Broth (MHB, Roth) to concentration
220 of 1×10^6 bacteria/ml. Two-fold serial dilutions of vancomycin in 50 μ l of MHB were prepared
221 in 96-well polypropylene plates with starting concentration of vancomycin set to 1 mg/ml.
222 Afterwards, 50 μ l of bacterial suspension was pipetted into each well. Bacterial growth was
223 measured with the use of a Tecan plate reader for 16 h with 30 seconds of vigorous shaking

224 and OD₆₀₀ measurement every 15 minutes. For each strain 3 technical and biological
225 repetitions were performed. No-antibiotic and negative controls were included in each assay.

226

227 **2.9. RNA isolation and real-time quantitative PCR (qPCR)**

228 Overnight cultures of strains 5706 and 6203 and their isogenic mutants were grown to OD₆₀₀=
229 0.45-0.55. Next, 1 ml of bacteria was harvested and RNA was isolated by the RNAsnap
230 method (37). For each sample, 1 µg of RNA was digested with DNase I (Thermo Scientific)
231 according to the manufacturer's instructions and reverse transcription was performed with the
232 use of iScript cDNA Synthesis Kit (BioRad). qPCR was performed with the use of Universal
233 SYBR Green qPCR Supermix (BioRad) and CFX96 Thermocycler (BioRad). Primers used
234 for qPCR are listed in Table 2. The qPCR data analysis was performed with CFX Manager
235 3.1 (BioRad) by normalizing the expression of *dfsA* and *sanA* genes to the reference gene
236 *tufA* with $\Delta\Delta C_q$ method (38).

237 **2.10. Gentamycin protection assays**

238 Adhesion and invasion assays were performed on strains generated in section 2.8. We chose
239 isolates with an altered infection phenotype. Here, we compared isolates with high infectivity
240 (=one phenotype) with isolates with low infectivity (=another phenotype) within one serovar.
241 As such altered infection phenotypes were similarly identified for Caco-2, IPEC-J2 and
242 CHIC-8E11 cells, and further work was done only with the Caco-2 cell line.

243 All bacteria were previously determined to be susceptible to gentamycin at a concentration of
244 50 µg/ml. First, O/N cultures of bacteria (grown for 16 h) in 1 ml of LB in 2-ml Eppendorf
245 tubes were diluted to OD₆₀₀=0.05 in 5 ml of LB in 15-ml falcon tubes and grown to
246 OD₆₀₀=2.0. Next, bacteria were washed once in PBS and resuspended in antibiotic-free cell
247 culture medium. Bacteria (1.2×10^7) in 1 ml of medium was added to Caco-2 cells per well
248 (proportional to the concentration of bacteria in infection assays in a 96-well plate format).

249 After 1 h incubation of bacteria with cells, bacterial suspension was discarded, cells were
250 washed three times with 1 ml of PBS and new medium with or without gentamycin was
251 added. After 1 h of incubation, supernatants were discarded and cells were washed three times
252 with PBS. Cell monolayers were lysed with 1% of Triton-X 100 (Sigma) in PBS, dilution
253 series in PBS were made and bacteria were spread on LB agar plates. Next morning, colony-
254 forming units were counted. For each strain at least 3 technical and biological repetitions were
255 performed.

256 2.11. RNA sequencing and differential transcriptome analysis

257 Four *S. Typhimurium* and four *S. Enteritidis* isolates with lowest and highest infectivity were
258 selected for RNA sequencing. Overnight cultures were diluted 100x in 10 ml of LB in 50-ml
259 Falcon tubes and grown to $OD_{600}=0.4$. Next, 5 ml of bacterial culture was added to 10 ml of
260 RNeasy Protect Bacteria (Qiagen), vortexed 10 sec. and incubated at room temperature for 5 min.
261 Next, all tubes were centrifuged 4,400xg for 15 min. at room temperature, supernatants were
262 discarded and bacterial pellets were frozen at -80°C (the pellet was stored and used for no
263 longer than 1 week).

264 RNA isolation was done with RNeasy Mini Spin Kit (Qiagen). Next, RNA was digested with
265 RNase-free DNase (Qiagen). RNA quantity and purity was checked with Nanodrop and RNA
266 quality was determined with Agilent 2100 Bioanalyzer.

267 RNA was sequenced with the use of HiSeq X platform at Sanger Institute Sequencing
268 Facility. Library for sequencing was prepared with 'NEB Ultra II RNA Custom Kit' (NEB).
269 Sequencing quality was assessed on the basis of average base quality, GC content and adapter
270 contamination (24). Reads were mapped to the reference genomes of *S. Typhimurium* SL1344
271 and *S. Enteritidis* P125109, respectively, and counted with the use of Rsubread package (39).
272 Differential expression analysis was performed with use of edgeR (40), and a gene was
273 considered as differentially express (DE) when FDR value was equal or lower than 0.05.

274

275 **2.12. Data processing and statistical analysis**

276 Statistical analysis was performed using R software (41). All figures were prepared with
277 the ggplot2 package implemented in the R software (42).

278 For screening of the 127 *Salmonella* isolates results from three experiments (in total nine
279 measurements) were baselined using results from empty-well controls. First, to eliminate the
280 inter-replicate variance, median infection value (bacteria/mm²) for each of three replicates
281 from one measurement was computed. Next, the final infection value (bacteria/mm²) was
282 computed as a median of medians coming from three experiments. To facilitate comparison
283 between measurements from different plates, final median values were standardized using the
284 median value of SL1344 strain for a given plate. The procedure was repeated for three cell
285 lines and both incubation periods. Standardized data were later compared using Mood test
286 with Benjamini-Hochberg correction for multiple comparisons. Statistical analysis for growth
287 curves and gentamycin protection assays was performed using Wilcoxon test implemented in
288 R software.

289 **2.13. Data availability**

290 Genome sequence and RNAseq data for *Salmonella enterica* have been deposited at the NCBI
291 under the BioProject ID PRJNA626643 and PRJNA667254, respectively.

292

293 **3. Results**

294 **3.1. *Salmonella* transformation**

295 Bacterial isolates were transformed with a GFP-expressing plasmid for automated
296 quantification by our VideoScan method. To do this, susceptibility to ampicillin and
297 kanamycin was first assessed for 128 *Salmonella* isolates. Most isolates were sensitive to both
298 antibiotics, one isolate was resistant to kanamycin, and 37 were resistant to ampicillin. A single
299 isolate was resistant to both ampicillin and kanamycin and was therefore excluded from

300 further work. Due to the high number of ampicillin-resistant isolates, the antibiotic resistance
301 cassette in plasmid pFPV25.1 was exchanged from ampicillin to kanamycin to create plasmid
302 pFPV25.1Kan. The remaining 127 isolates were successfully transformed with this new
303 plasmid.

304

305 **3.2. Quantification of *Salmonella* with VideoScan**

306 Before proceeding to automated quantification of infectivity of the 127 GFP-expressing
307 isolates, the reliability of the fluorescence microscope-based VideoScan method was assessed.
308 Serial dilution experiments were performed with *S. Typhimurium* SL1344 to determine the
309 dynamic range of the VideoScan. The quantification of *Salmonella* was possible within a
310 wide range of bacterial dilutions for all three cell lines tested (Fig. 1). The linear range of the
311 assay was 2×10^4 - 2.5×10^7 ($R^2=0.96$), 2×10^4 - 3×10^7 ($R^2=0.96$) and 2×10^4 - 3×10^7 ($R^2=0.92$) for
312 IPEC-J2, Caco-2 and CHIC-8E11, respectively. Thus, quantification of GFP-labelled
313 *Salmonella* was reliable within the tested serial dilution range. A bacterial concentration of
314 2×10^6 bacteria/well (4×10^7 /ml) was chosen for further assays.

315

316 **3.3. Cell line infection screening revealed no link between cell type and source of** 317 **host-specialist *Salmonella* isolates**

318 Porcine (IPEC-J2), human (Caco-2) and chicken (CHIC-8E11) intestinal epithelial cells were
319 infected with the 127 GFP-expressing *Salmonella* isolates for 1 and 4 h. Bacteria which
320 infected cells were counted using the VideoScan and data were fitted to an appropriate
321 statistical model (Fig. 2).

322 Surprisingly, *Salmonella* host-specialists did not appear to be more infective in cell lines
323 originating from the host species they were isolated from. *S. Choleraesuis* (pig specialist)
324 showed 12.5 times lower infection rates in porcine IPEC-J2 cells than Caco-2 ($p<0.01$),

325 whereas *S. Gallinarum* (chicken specialists) showed the highest infection in these cells, and
326 this result was 20 times higher than infection in CHIC-8E11 cells ($p < 0.001$). Also, *S.*
327 Typhimurium isolated from humans did not show greater infection of human Caco-2 cells.
328 Similar results were found using *S. Enteritidis* from humans or chicken and *S. Typhimurium*
329 from pigs. Among all *Salmonella* groups, *S. Gallinarum* was 50-100 times less infective than
330 other groups on CHIC-8E11 ($p < 0.001$) and *S. Choleraesuis* was 1.2-28 times more infective
331 than other groups on Caco-2 (statistically significant only for comparison with *S. Gallinarum*,
332 $p < 0.001$). In general, CHIC-8E11 cells were most susceptible to *Salmonella* infection
333 ($p < 0.01$), while IPEC-J2 were most resistant ($p < 0.01$) (Supplementary Table 2).
334 When the increase of infecting bacteria was calculated between the two incubation times
335 ($\Delta\text{Inf} = \text{Inf}_{4\text{h}} - \text{Inf}_{1\text{h}}$), *S. Choleraesuis* had the highest median increase (1.2-33-fold) on Caco-2
336 cells when compared with other groups (statistically significant only for comparison with *S.*
337 *Gallinarum*, $p < 0.001$) (Supplementary Fig. 1, Supplementary Table 3). The highest ΔInf on
338 CHIC-8E11 cells was observed for *S. Dublin* (1.2-78-fold higher, statistically significant for
339 comparison with *S. Gallinarum* and *S. Enteritidis* human, $p < 0.01$) and on IPEC-J2 for *S.*
340 *Typhimurium* isolated from humans (1.3-8.3-fold higher, statistically significant in
341 comparison with *S. Choleraesuis*, *S. Dublin*, *S. Enteritidis* from chicken and *S. Enteritidis*
342 from human, $p < 0.01$). Nearly all *Salmonella* groups had the lowest ΔInf in IPEC-J2 with the
343 exception for *S. Gallinarum*, which had the highest ΔInf in this cell line ($p < 0.01$).

344

345 **3.4. Analysis of *Salmonella* genomes revealed the presence of natural mutations**

346 **responsible for altered infection phenotypes**

347

348 To identify bacterial factors responsible for varying infectivity, the genomes of 30 isolates
349 with the highest and lowest infectivity were sequenced and compared. The pangenome

analysis revealed no differences in gene content (i.e. gene presence or absence) associated with infection phenotype between highly infective isolates in comparison to marginally infective isolates. To determine the similarity among isolates with altered infection phenotypes, core genome-based and additional binary genome-based phylogenetic trees were constructed (Fig. 3, Supplementary Fig. 2). Moreover, MLST sequence types (ST) and the presence of pSV plasmids which are common in almost all serovars were determined. Highly infective isolates did not form separate subclades/groups and did not belong to different STs. Presence of pSV plasmids did not correlate with infectivity levels.

All the results at this stage of work lead to the hypothesis that altered infection phenotypes were driven not by gene content, but rather sequence variation in *Salmonella* genomes. Therefore sequencing reads were mapped to reference genomes and aligned reads were compared to identify any gene mutations in comparison to the reference genome. Fourteen candidate genes were found to be potentially associated with altered infection phenotypes and are listed in Table 3 alongside with function, description of mutation and whether similar mutations could be found in genomes in the GenBank database. The functions of these 14 candidate genes were: 1) virulence gene expression regulation (e.g. *dksA*), 2) synthesis of cell wall or membrane elements (e.g. *rfaL*), 3) and cell membrane components (e.g. *ompD*). The role of several of these genes in *Salmonella* adhesion and invasion of cells has not been investigated so far (e.g. *sanA* and *yidR*). Natural mutations found in the strains with altered infection phenotypes included deletions, and single nucleotide substitution which resulted in premature stop codons or amino acid alterations. Alleles with these natural mutations were found for six genes in GenBank Reference Genomes database with BLASTN search.

Functional assays confirm the role of *dksA* and *sanA* in host cell infection

374 To confirm the contribution of mutations to altered infection phenotypes, two genes were
375 selected and genetically manipulated for further elucidation of their functions: *dksA* and *sanA*
376 (the function of each gene is summarized in Table 3). To study the effect of mutations found
377 in this work, *dksA* and *sanA* in *S. Enteritidis* clinical isolates (no. 5706 and 6203) were
378 replaced with the wild-type sequences from *S. Enteritidis* PT4 strain no. P125109. No obvious
379 phenotypic changes in comparison to parental strains were observed in the complemented
380 strains, indicating that the genetic manipulations did not affect bacterial morphology. Isogenic
381 strains of isolates 5706 and 6203 were tested for *dksA* and *sanA* expression, respectively.
382 Relative expression of wildtype *dksA* was 40% lower than the *dksA* mutated variant ($p =$
383 0.001). For 6203 isogenic strains, there was no difference in expression between wildtype and
384 mutated *sanA* variants.

385 Gene *dksA* encodes a stringent response regulator required for growth in minimal medium,
386 therefore the isogenic strains of isolate 5706 were tested for growth in M9 minimal medium
387 supplemented with glucose. The strain expressing wildtype DksA had 1.7-1.9 faster growth
388 rate and doubling time in exponential phase of growth as compared to the strains expressing a
389 DksA mutated variant ($p < 10^{-5}$, Figure 4 A, Supplementary Table 4). Subsequently, the ability
390 to adhere to and invade Caco-2 cells was evaluated and the wildtype strain had 80 % higher
391 infection and 30 % higher invasion levels compared to the strains with a *dksA* mutated variant
392 ($p = 0.05$) (Figure 4 B).

393 The *sanA* gene deletion in *E. coli* has been linked to increased susceptibility to
394 vancomycin. The function of this gene is often studied *in vitro* by vancomycin susceptibility
395 testing. Therefore, isogenic strains of isolate 6203 with wildtype and mutated variant of *sanA*
396 were tested for growth in vancomycin. Strains carrying the mutated variant were more
397 resistant to vancomycin than strain carrying the wildtype *sanA*. Strain with wildtype *sanA*
398 gene showed growth in up to 31.25 $\mu\text{g/ml}$ vancomycin, whereas strains with a mutated *sanA*

399 survived concentrations up to 62.5 µg/ml. Next, strains carrying isogenic variants of *sanA*
400 were tested in adhesion and invasion assays. Strains carrying a mutated variant of *sanA*
401 infected cells 10 % higher and invaded 20 % higher than the strain carrying wildtype *sanA*
402 variant, but the statistical analysis revealed that these differences are not significant (Figure
403 4C).

404

405 **3.5. Different expression levels of T3SS are responsible for altered infection**

406 **phenotypes in *S. Typhimurium* and *S. Enteritidis* isolates**

407

408 Based on the results from the RNAseq and transcriptome studies, the number of differentially
409 expressed (DE) genes between isolates varied from 15 to 67 in the case of *S. Enteritidis* and
410 from 48 to 324 for *S. Typhimurium* isolates (Supplementary Table 5). All DE genes were
411 grouped by biological processes they represent and this analysis revealed the presence of two
412 gene groups- “Bacterial secretion system” and “Bacterial invasion of epithelial cells”-
413 associated with invasion of epithelial cells (Supplementary Figure 3A and 3B). These genes
414 belong to the T3SS injectosome machinery or are secreted into epithelial cells and mediate
415 bacterial internalization. Two *S. Typhimurium* isolates representing the high-infecting
416 phenotype (isolate no. 5727, 6191) had from 2- to 280-fold increased expression of these
417 genes in comparison to two isolates with a low-infecting phenotype (isolate no. 5735, 6185)
418 (Figure 5). This was not the case for *S. Enteritidis*, as one *S. Enteritidis* isolate (isolate no.
419 6203) with low-infecting phenotype had expression profiles similar to two *S. Enteritidis*
420 isolates with high-infecting phenotypes, indicating a contribution of other factors to the
421 adhesion and invasion process.

422 **4. Discussion**

423 This study was aimed at identifying new genes with possible contributions to adhesion
424 and/or invasion by *Salmonella* with host epithelial cells. To develop a high-throughput
425 screening method for the quantification of *Salmonella* in cell culture infections, we used the
426 VideoScan Platform developed used in our lab for nucleic acid detection or quantification, *E.*
427 *coli* quantification in cell culture adhesion assays, quantification of bacterial adhesion to
428 proteins, and automated detection of autoantibodies in cell-based assays (23, 43–45). The new
429 VideoScan module leverages the detection of and screening of adherent and invasive GFP-
430 expressing bacteria to provide an output of bacterial numbers adhering to and invading a cell
431 line. This assay has advantages over other bacterial quantification methods in that it is
432 simpler, avoids complicated staining procedures involving membrane permeabilization,
433 avoids the often laborious selection of suitable antibodies for different serovars and problems
434 arising from non-specific antibody binding, and there is no need to perform dilution series and
435 CFU determinations on agar plates (46). The only disadvantage of this approach is the
436 requirement for bacterial transformation with a plasmid encoding the GFP protein, where it
437 could be difficult to find an antibiotic selection marker for antibiotic-resistant isolates (47).

438 This approach was used to test if 127 *Salmonella* isolates, representing serovars with
439 different host ranges, were better at infecting cells originating from the associated host.
440 However, using this model this was shown not to be the case. This might be because there are
441 many other host factors related to *Salmonella* host specificity, that were not present in our
442 simple model of epithelial cell infection (48).

443 In an effort to explain the differences in infectivity among isolates, we focused our efforts to
444 analyse and identify the possible underlying genetic explanations for the outcomes in our of
445 the cell line infection assays in isolates that showed altered infection phenotypes in
446 comparison to the majority of isolates screened in each group. Though *Salmonella* virulence
447 plasmid (pSV) was previously associated with septicaemia and enteritis (49), we were not

448 able to find any association between altered infection phenotype and the presence of pSV.
449 However, we were able to find naturally occurring mutations present in the genomes of
450 clinical isolates of *Salmonella*. Interestingly, a majority of the mutations we found were not
451 associated with any factors directly responsible for invasion or adhesion. We did not identify
452 any mutations in genes encoding T3SS, Rck or PagN that would explain the altered infection
453 phenotypes, nor in genes encoding adhesins, except for a single base substitution in the *fimH*
454 gene leading to premature translation termination in one of the isolates with a high-infection
455 phenotype. Usually, loss of type 1 fimbriae (T1F) expression (*fimH* gene codes for tip adhesin
456 of T1F), which this deletion confers, generally results in lower cell adhesion (50).
457 Noteworthy is the fact that bacteria for adhesion assays with T1F are usually grown under
458 different conditions from those used in our screens (12, 51).

459 When we looked at the function of genes with natural mutations, we noticed that they
460 are related to cell membrane/surface structures and cell wall or surface structure expression
461 regulation. There are reports which link pseudogene formation in cell membrane/surface
462 structures with host adaptation (52, 53). These could explain the presence of natural mutations
463 in host-restricted (*S. Gallinarum*) and host-adapted serovars (*S. Dublin* and *S. Choleraesuis*).
464 Other explanations for natural mutations could be that these isolates were isolated from
465 asymptomatic carriers and the presence of natural mutations might increase long-term
466 survival within the host without recognition by the immune system. Similar findings were
467 ascertained by Klemm et al., (2016), where the formation of pseudogenes in membrane
468 proteins have been reported in *S. Enteritidis* isolates of one strain sampled multiple times over
469 15 years from one patient (54). Unfortunately, we had no information about the host's clinical
470 status at the time of sampling, and therefore we cannot confirm or rule out this possibility.
471 Another possibility that could explain the occurrence of altered infection phenotypes and
472 natural mutations is the mutagenic properties of antibiotics or other xenobiotics. Multiple

473 studies have shown that various classes of antibiotics can induce stress and mutations at
474 subinhibitory concentrations (55–57). These mutations are the effect of adaptive bacterial
475 response to antimicrobials or a consequence of direct interaction of antibiotics with bacterial
476 DNA (58). When we checked for the presence of antibiotic resistance genes, nine out of 30
477 *Salmonella* isolates had at least one antibiotic resistance gene, confirming a possible
478 exposure to antibiotics. Antibiotic-induced mutations have a deleterious effect, such that the
479 bacteria bearing them would have decreased fitness and more negative selection in
480 comparison to wildtype isolates. This negative selection could perhaps explain why the
481 natural mutations that we observe in our strain collection can be rarely found in GenBank. To
482 confirm this hypothesis in future, an antibiotic-driven mutant library could be created and
483 tested for infection of epithelial cells.

484 Contribution of mutations to altered infection phenotype was functionally validated
485 for two genes: *dksA* and *sanA*. Gene *dksA* was selected because its contribution to host cell
486 adhesion and invasion was known. Little was known about the influence of *sanA* on
487 *Salmonella* host cell adhesion, therefore they were chosen for further investigation.
488 Complementation of the mutation found in *dksA* led to an increase in growth rate in minimal
489 medium and host cell infectivity. Though the importance of the amino acid at position 88 in
490 DksA for growth in minimal media has been described previously (59), a link to cell line
491 invasion has not been described so far. Deletion of *sanA* was previously associated with
492 increased susceptibility to vancomycin in *E. coli* (60). One of our isolates with altered
493 infection phenotypes had a 10 nucleotide deletion in *sanA*. We therefore exchanged the
494 mutation with the wild-type sequence and determined whether this would lead to rescue of
495 certain functions. Indeed, complementation of the mutation led to a decrease in resistance to
496 vancomycin, and further influenced adhesion and invasion. Though the mode of action for
497 *dksA* in adhesion and invasion was described for *S. Typhimurium* in previous studies (59, 61,

498 62), there is no information about the role or possible contribution of *dksA* sequence variation
499 and *sanA* to bacterial adhesion or invasion.

500 We decided to get a global overview of expression profiles for the isolates with altered
501 infection phenotypes, so we conducted a transcriptomic analysis, which highlighted the
502 importance of T3SS expression levels in the determination of an isolate's infection phenotype.
503 Similar differences were observed in the study of Shah (2014), where 38 genes from SPI-1
504 showed reduced expression in less virulent *S. Enteritidis* isolates in comparison to more
505 virulent *S. Enteritidis* (63). Three isolates with low infection phenotype included in this
506 analysis had mutations in *aroA*, *ompD*, *yidR* and *sirA*. Though the contribution of *aroA* and
507 *sirA* in the regulation of genes associated with T3SS has been shown previously and there is
508 information about a possible connection between T3SS and *ompD* expression levels (64–66),
509 there is so far no information about the *yidR* gene. One strain with low infection phenotype
510 and a mutation in the *sanA* gene had a gene expression profile similar to highly infecting
511 phenotype. Since knowledge about this gene is scant, an explanation for this observation
512 requires further study.

513 Our analysis of genomes and transcriptomes of the isolates with altered infection phenotypes
514 allowed us to find explanations for lowered infection of epithelial cells. However, we were
515 not able to identify the factors contributing to highly infecting phenotypes of the selected
516 *Salmonella* isolates. It is possible that other factors, like methylation, influence higher
517 virulence of these isolates, as shown before in the case of *Salmonella* (67). A detailed
518 investigation of these additional factors is out of the scope of present investigation.

519 To summarize, this work was an exploration of what we can learn from the observing the
520 interaction of clinical isolates of *Salmonella* with host cells of intestinal epithelial origin. We
521 have shown that the majority of isolates have similar infection phenotypes and isolates with
522 altered infection phenotypes provide a source for determination of new genes or new gene

523 variants influencing epithelial infection, including novel factors associated with adhesion and
524 invasion of epithelial cells by *Salmonella*. We identified two genes, *sanA* and *yidR*, that were
525 not previously associated with adhesion or invasion of host cells. We consider these genes to
526 be interesting candidates for further investigations within the context of *Salmonella* virulence.
527 Our study reveals new questions regarding the origin of such mutations and their contribution
528 to pathogen evolution and pathogenicity, and suggest that the approach used here with larger
529 strain collections belonging to the same serovar, clinical data and the use of genome-wide
530 association studies could provide important insights into *Salmonella* host cell interactions.

531

532

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538

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745 Is Essential for Type III Secretion System Expression and Virulence of *Salmonella*
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752 Table 1. List of bacteria used in this study

Strains	Description (Group name)	Reference numbers	Source
<i>Escherichia coli</i> XL1Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB lacIq ZΔM15 Tn10 (Tetr)]</i>		Agilent Technologies
<i>Salmonella enterica</i> subsp. <i>enterica</i> serovar Typhimurium	20 strains, isolated from human diarrhea cases (Typhimurium-human)	5717-5725, 6181- 6191	Mydlak/Thoraus Diagnostic Laboratory, Cottbus
<i>Salmonella enterica</i> subsp. <i>enterica</i> serovar Typhimurium	20 strains, isolated from porcine faeces samples (Typhimurium-pig)	5726-5745	BfR, Berlin
<i>Salmonella enterica</i> subsp. <i>enterica</i> serovar Enteritidis	20 strains, isolated from human diarrhea cases (Enteritidis-human)	5702-5710, 6170-6180	Mydlak/Thorasch Diagnostic Laboratory, Cottbus
<i>Salmonella enterica</i> subsp. <i>enterica</i> serovar Enteritidis	14 strains, isolated from chicken faeces samples (Enteritidis-chicken)	6192- 6195,6197, 6198, 6200- 6207	German Federal Institute for Risk assessment (BfR), Berlin
<i>Salmonella enterica</i> subsp. <i>enterica</i> serovar Gallinarum	19 strains, isolated from chicken faeces samples (Gallinarum)	5766-5771, 5773-5785	BfR, Berlin
<i>Salmonella enterica</i> subsp. <i>enterica</i> serovar Dublin	20 strains, isolated from bovine faeces samples (Dublin)	5746-5765	BfR, Berlin
<i>Salmonella enterica</i>	15 strains, isolated from	6053, 6054,	BfR, Berlin

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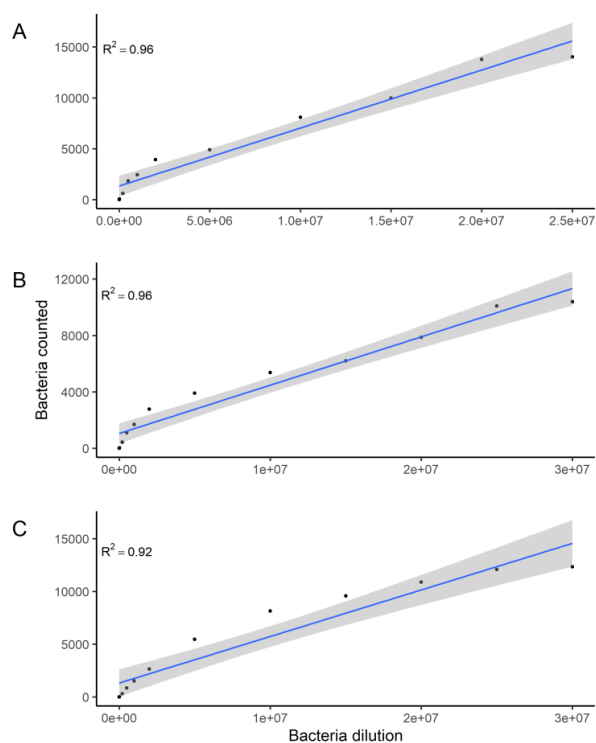
subsp. <i>enterica</i> serovar Choleraesuis	various sources-porcine faeces, wild boars, meat products, reptile (Choleraesuis)	6138-6150	
<i>Salmonella enterica</i> subsp. <i>enterica</i> serovar Typhimurium SL1344			Free University, Berlin

759 Table 2.
760 List of primers used in this study.

Primer name	Primer sequence (5'→3')	Gene	Source
pFVP25.1KmRev	CTGTCAGACCAAGTTTACTCATATATAC	<i>kanR</i>	This study
KanpFVP25Fwd	TGAGTAACTTGGTCTGACAGTCAGAAGAACTCGTCAAGAAG	<i>kanR</i>	This study
KanpFVP25Rev	TAATATTGAAAAAGGAAGAGTATGATTGAACAAGATGGAATTG	<i>kanR</i>	This study
pFVP25.1KmFwd	ACTCTTCCTTTTCAATATTATTG	<i>kanR</i>	This study
dksAdelNEGfor	ATGCAAGAAGGGCAAAACCGTAAAACATCGTCCCTGAGT ATTCTCGCCAT GCTTGCAGTGGGCTTACATG	<i>dksA</i>	This study
dksAdelNEGrev	TTAACCCGCCATCTGTTTTTCGCGAATTTTCAGCCAGCGTTT TGCAGTCGA TCAGAGCAGGATCGACGTCC	<i>dksA</i>	This study
dksAfor	ATGCAAGAAGGGCAAAACCG	<i>dksA</i>	This study
dksArev	TTAACCCGCCATCTGTTTTTCG	<i>dksA</i>	This study
dksA100UpstreamFor	ACAGGGTTGTCAAGTGTTACG	<i>dksA</i>	This study
dksA100DownstreamRev	TAACGAGCCGAAATGCAGTTC	<i>dksA</i>	This study
dksAinternalRev	AGTGCGATCGACTTCATCC	<i>dksA</i>	This study
k2	CGGTGCCCTGAATGAACTGC	<i>kanR</i>	(68)
sanAdelNEGfor	ATGTTAAAGCGCGTGTTTTACAGCCTGTTGGTCCTGG TAGGCTTGCTGCT GCTTGCAGTGGGCTTACATG	<i>sanA</i>	This study
sanAdelNEGrev	TCATTTCCCTTTTTCTTTTCCAGTTCAAGCAATTGTT CCGGCGTAACTG TCAGAGCAGGATCGACGTCC	<i>sanA</i>	This study
sanAfor	ATGTTAAAGCGCGTGTTTTAC	<i>sanA</i>	This study

			y
sanArev	TCATTTCCCTTTTTCTTTTCCAG	<i>san</i> <i>A</i>	This stud y
sanA100Upstream For	CGATACAAGGGAAATCATGCTG	<i>san</i> <i>A</i>	This stud y
sanA100Downstre amRev	TTCCAGGCCTCACGGAAG	<i>san</i> <i>A</i>	This stud y
sanAinternalRev	GCCCTGGATACGATAACGA	<i>san</i> <i>A</i>	This stud y
tufAqPCRfor	TGTTCCGCAAAGCTGCTGGACG	<i>tuf</i> <i>A</i>	(69)
tufAqPCRrev	ATGGTGCCCGGCTTAGCCAGTA	<i>tuf</i> <i>A</i>	(69)
dksAqPCRfor1	TGAAGCATGGCGTAATCAACTC	<i>dks</i> <i>A</i>	This stud y
dksAqPCRrev1	TCCAGGCTAAACTCCTCTTCC	<i>dks</i> <i>A</i>	This stud y
sanAqPCRfor1	AAACAGCGCCCTATATCTATGAC	<i>san</i> <i>A</i>	This stud y
sanAqPCRrev1	TGATTAATGACACCCTTGCGA	<i>san</i> <i>A</i>	This stud y

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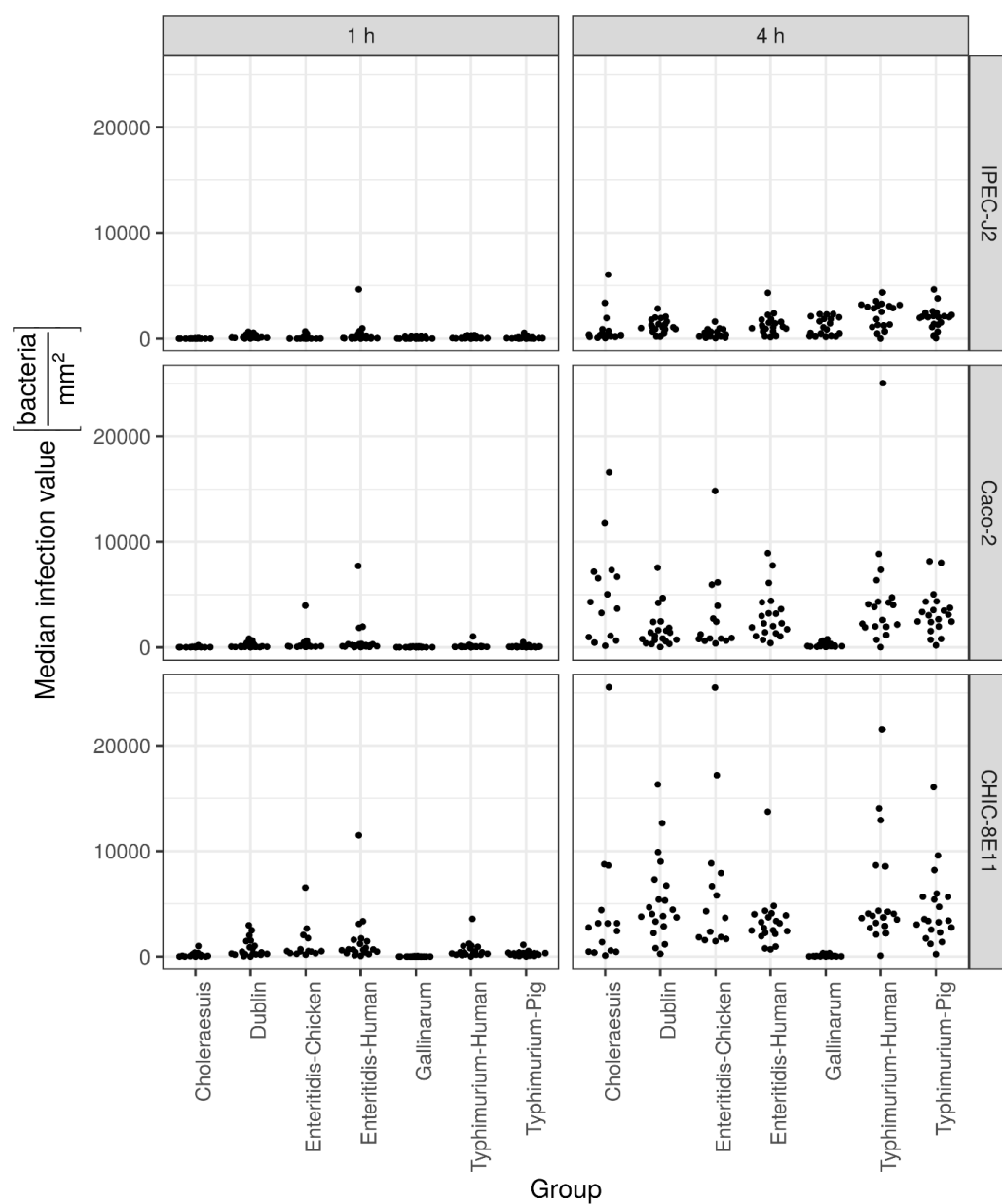


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763 **Figure 1. Dilution series of *Salmonella* in cell line infection assays.**

764 This assay was performed to determine the linear range of GFP-expressing *S. Typhimurium*
765 SL1344 in infection assays with IPEC-J2 (A), Caco-2 (B) and CHIC-8E11 (C) cell lines in a 96-
766 well plate format for 1 h. Images were automatically taken by the VideoScan instrument. Black
767 dots represent a median value from three assays in triplicates for each bacterial dilution. Blue
768 lines connecting dots are smoothed trend lines and grey areas around the lines are 0.95
769 confidence interval of the linear regression.

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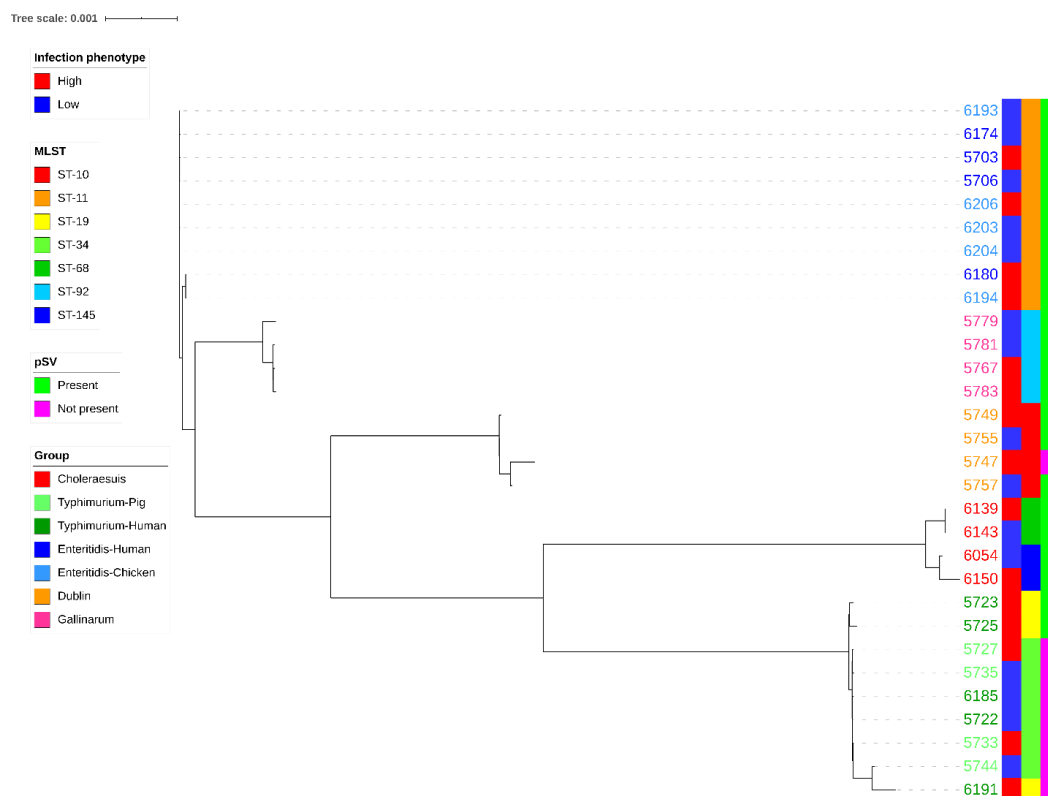
772 **Figure 2. *Salmonella* infection of IPEC-J2, Caco-2 and CHIC-8E11 cell lines.**

773 127 *Salmonella* isolates from *S. Typhimurium* of human (Typhimurium-Human) and pig

774 (Typhimurium-Pig) origin, *S. Enteritidis* of human (Enteritidis-Human) and chicken (Enteritidis-

775 Chicken) origin, *S. Choleraesuis* (Choleraesuis), *S. Dublin* (Dublin), *S. Gallinarum* (Gallinarum)
776 were compared for IPEC-J2, Caco-2 and CHIC-8E11 cell line infection after 1 h and 4 h of
777 incubation. Each dot represents a median infection value from three measurements in triplicates
778 for one strain. Results are shown as number of bacteria per mm².

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781 **Figure 3. Core genome tree of *Salmonella* with different infection phenotypes**

782 Annotated genomes were analysed with Roary. Next, the core genome alignment of 3473 genes
783 from 30 genomes was used to generate a tree with FastTree 2.1. Information about infection
784 phenotype, group, MLST sequence types and presence of pSV plasmid were added with the use
785 of iTOL. Groups and isolate numbers used in analysis: Choleraesuis- 6054, 6139, 6143, 6150;
786 Typhimurium-Pig- 5727, 5733, 5735, 5744; Typhimurium-Human- 5722, 5723, 5725, 6185,

6191; Enteritidis-Human- 5703, 5706, 6174, 6180; Enteritidis-Chicken- 6193, 6194, 6203, 6204,

6206, Dublin- 5747, 5749, 5755, 5757; Gallinarum- 5767, 5779, 5781, 5783.

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Table 3. Candidate genes responsible for altered infection phenotype with a description of their

function (where possible), mutation and presence of genomes with the same changes in the

GenBank database (GenBank query was made with use of RefSeq Genomes Database).

Abbreviations: NN- nucleotide, AA- amino acid, Tym- *S. Typhimurium*, cds- coding sequence

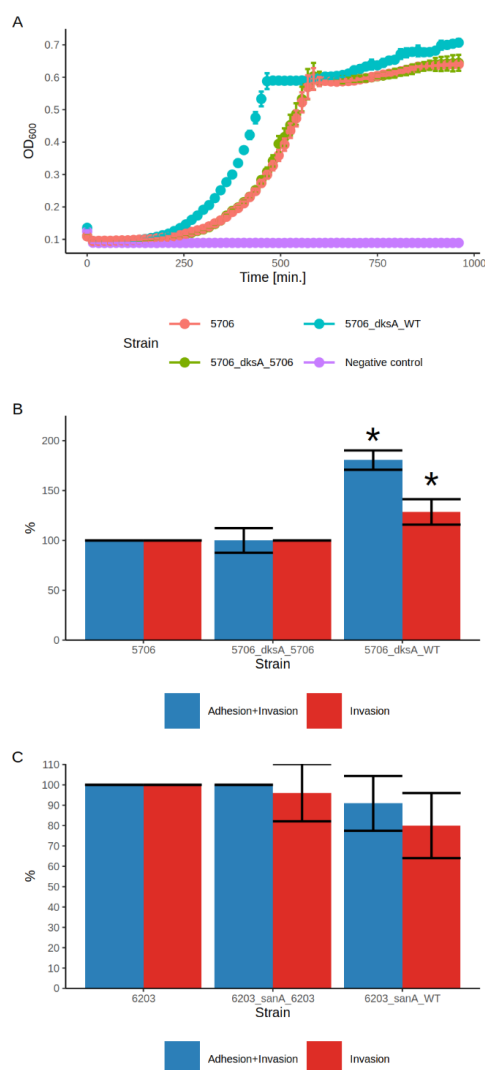
Serovar	Gene	Function	Mutation	GenBank query*
<i>S. Enteritidis</i>	<i>dksA</i>	Required for growth in minimal medium and intestinal colonization; mediates adaptation to oxidative and nitrosative stress.	Two AA deletion at position 87 and 88 in isolate 5706	Yes (54 sequences, 14 sequences with mutations in the same region)
<i>S. Enteritidis</i>	<i>ompD/nmpC</i>	Porin OmpD, mediates binding of Tym to T84 and U937 cells	Deletion of starting 300 bp of cds in isolate 6174	No
<i>S. Enteritidis</i>	<i>yidR</i>	Putative ATP/GTP-binding protein; mutant has reduced binding to/persistence in lettuce.	Stop codon at position 317 in isolate 6174	Yes (4 genomes, 21.10.2019)
<i>S. Enteritidis</i>	<i>snaA</i>	Vancomycin high-temperature exclusion protein; required for bile tolerance in <i>S. Typhi</i> .	Deletion of 10 NN in isolate 6203	Yes (1 genome, 14 NN deletions in the same region, 21.10.2019)

<i>S. Enteritidis</i>	<i>rfaL (waaL)</i>	O-antigen ligase; in Tym affects motility.	12 NN deletion in isolate 6193	No (21.10.2019)
<i>S. Typhimurium</i>	<i>sirA</i>	Invasion response-regulator; global regulator of genes mediating enteropathogenesis.	14 NN deletion in isolate 5735	Yes (2 genomes, 21.10.2019)
<i>S. Typhimurium</i>	<i>aroA</i>	3-phosphoshikimate 1-carboxyvinyltransferase; mutation leads to auxotrophy for aromatic amino acids.	78 NN deletion in isolate 6185	No (21.10.2019)
<i>S. Typhimurium</i>	<i>fimH</i>	Adhesin of type 1 fimbriae.	Stop codon at position 80 in isolate 6191	Yes (13 genomes, 21.10.2019)
<i>S. Gallinarum</i>	<i>lrhA</i>	Transcriptional regulator; negative regulator of flagella expression in early cell growth phases in Tym.	3 NN deletion (at 100 position), 1 AA (Ala), in isolate 5781	Yes (7 genomes, 21.10.2019)
<i>S. Gallinarum</i>	<i>rscD</i>	Phosphotransfer intermediate protein in a two-component regulatory system with RcsBC; is implicated in the control of capsule and flagella synthesis and biofilm formation.	5 NN deletion in isolate 5781	No
<i>S. Dublin</i>	<i>ompW</i>	Outer membrane protein W; mediates methyl viologen	1 NN deletion in isolate 5755, 5757	No

		(paraquat) efflux in Tym.		
<i>S. Dublin</i>	<i>mpl</i>	UDP-N-acetylmuramate:L-alanyl-gamma-D-glutamyl-meso-diaminopimelate ligase; peptidoglycan synthesis.	Deletion after codon 423 in isolate 5757	No
<i>S. Choleraesuis</i>	<i>rtsA</i>	Increases expression of the invasion genes by inducing <i>hilA</i> , <i>hilD</i> , <i>hilC</i> genes and the <i>invF</i> operon.	1 SNP that leads to change from Arg to Gln in isolate 6143.	No
<i>S. Choleraesuis</i>	<i>wza</i>	Polysaccharide (colanic acid) export protein Wza; involved in exopolysaccharide (EPS) production.	1 SNP in codon 171 that leads to change for stop codon in isolate 6054	No

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797 **Figure 4. Phenotypic assays for *dksA* and *sanA* variants.**

798 A) Growth curve of *S. Enteritidis* 5706 isogenic strains in M9 minimal medium supplemented
 799 with 0.2% glucose. Dots represent a median value from three assays in triplicates for each time
 800 point. B) Gentamycin protection assay with the use of *S. Enteritidis* 5706 isogenic strains and
 801 Caco-2 cells. Strains used in A) and B): 5706- isolate with mutated *dksA* variant,

5706_dksA_5706- isolate with mutated *dksA* variant introduced by negative selection system,
5706_dksA_WT- isolate with wildtype *dksA* variant introduced by negative selection system.
C) Gentamycin protection assay with the use of *S. Enteritidis* 6203 isogenic strains and Caco-2
cells. Strains used in C) 6203- isolate with mutated *sanA* variant, 6203_sanA_6203- isolate with
mutated *sanA* variant introduced by negative selection, 6203_sanA_WT- isolate with wildtype
sanA variant introduced by negative selection. Error bars represent the median absolute deviation.
Statistically significant results in B) were marked with “*” (p=0.05).

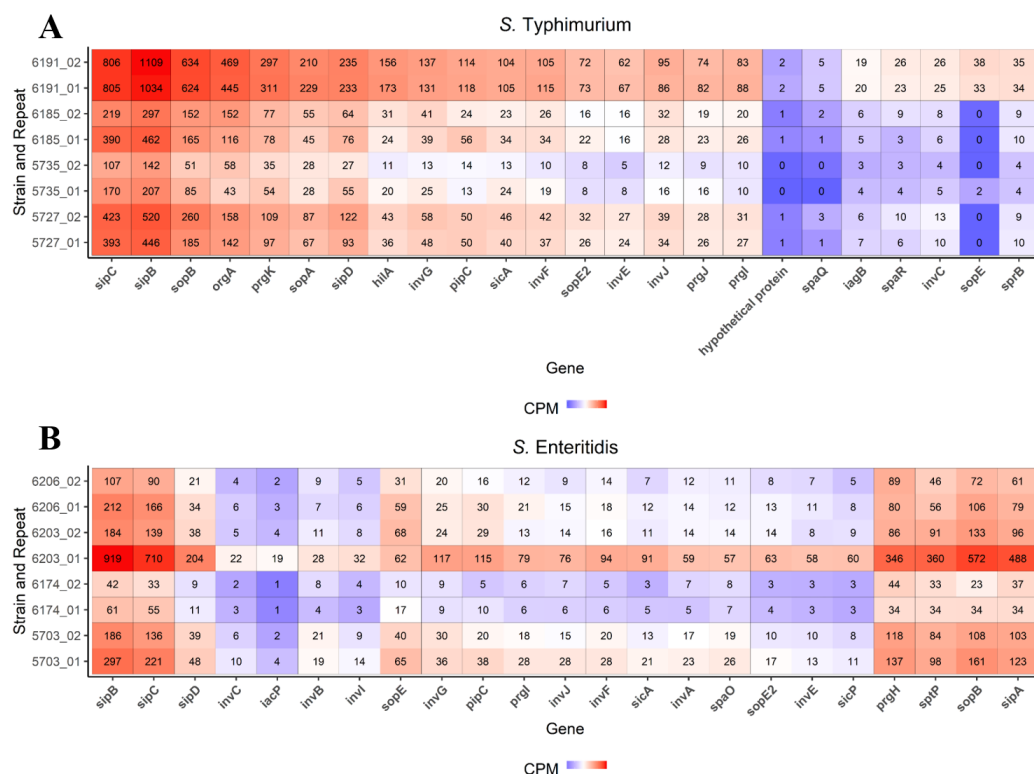


Figure 5. Differentially expressed genes associated with epithelial cell invasion.

812 Heatmap with expression levels of genes associated with invasion of epithelial cells in four *S.*
813 Typhimurium (A) and four *S. Enteritidis* isolates (B). Strain number and repeat are shown on the
814 y-axis. Isolates 5703, 5727, 6191 and 6206 represent high-infecting cell line phenotypes while
815 isolates 5735, 6174, 6185 and 6203 represent low-infecting phenotypes. Names of genes DE
816 expressed are shown on the x-axis. Color gradient is proportional to counts per million (CPM) for
817 each gene in each repeat. Number of CPM for each gene and repeat are written in rectangles.